Journal of Pathology
J Pathol 2005; **207**: 396–401
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1861

Original Paper

OCT-1 is over-expressed in intestinal metaplasia and intestinal gastric carcinomas and binds to, but does not transactivate, CDX2 in gastric cells

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Received: 10 May 2005
Revised: 18 July 2005
Accepted: 27 July 2005

Abstract

Intestinal metaplasia (IM) is a preneoplastic lesion of the stomach in which there is transdifferentiation of the gastric mucosa to an intestinal phenotype. The *caudal*-related homeobox gene *CDX2* encodes an intestine-specific transcription factor crucial for the regulation of proliferation and differentiation of intestinal cells. In addition, CDX2 is involved in the induction of IM in the stomach. The aim of this study was to access the putative involvement of OCT-1 in the induction of CDX2 expression *de novo* in gastric mucosa leading to the onset of IM. OCT-1 protein expression was evaluated by immunohistochemistry in 31 biopsies with chronic gastritis, 15 biopsies with foci of IM and adjacent gastric mucosa and 42 gastric carcinomas. Furthermore, we evaluated OCT-1 binding by electrophoretic mobility shift assay and activation of the *CDX2* promoter by co-transfecting a *CDX2* promoter/reporter construct with an OCT-1 expression vector in two gastric carcinoma cell lines, GP220 and MKN45. Our results show that OCT-1 is expressed in chronic gastritis, particularly when it is adjacent to IM and is expressed in 87% of IM foci. Furthermore, 74% of the gastric carcinomas were positive for OCT-1 and a strong association was observed between OCT-1 expression and intestinal-type carcinoma. We identified that OCT-1 binds to the *CDX2* promoter, although we could not see a transactivation effect in gastric carcinoma cell lines. In conclusion, we observed increased OCT-1 expression in IM and in intestinal gastric carcinomas and identified the capacity of OCT-1 to bind to the *CDX2* promoter, although we could not demonstrate a direct effect of OCT-1 in the transactivation of *CDX2*.

Keywords: intestinal metaplasia; gastric carcinoma; Cdx2; Oct1; promoter regulation

Introduction

Intestinal metaplasia (IM) is a preneoplastic lesion of the stomach in which there is transdifferentiation of the gastric mucosa to an intestinal phenotype [1,2]. Epidemiological studies have shown that IM is significantly associated with an increased risk of gastric carcinoma: approximately 80% of gastric carcinomas occur in the setting of IM and the relative risk for cancer development in the presence of IM is 6.4 [3].

The *caudal*-related homeobox gene *CDX2* encodes an intestine-specific transcription factor crucial for the regulation of proliferation and differentiation of intestinal cells [4–6]. In the adult intestinal epithelium, CDX2 is expressed mainly in differentiated cells [5,6]. Moreover, when transfected into intestinal epithelial cell lines, CDX2 was shown to induce cell differentiation and apoptosis [7–9].

In previous studies, we and others have shown that IM of the stomach as well as a subset of gastric carcinomas express CDX2 and the intestinal mucin MUC2, in contrast to the normal gastric mucosa [2,10–13]. More recently, we and others have shown that CDX2 regulates the *MUC2* gene in gastric cells through direct binding to its promoter [14,15]. It was also demonstrated that CDX2 expressed ectopically in the stomach of transgenic mice is sufficient to induce intestinal differentiation of the gastric mucosa [16,17]. Hence, CDX2 is one of the most likely candidates to be involved in the induction of IM of the stomach. Little is known about the regulation of *CDX2* in the normal intestine and, to the best of our knowledge, the regulation of aberrant *CDX2* expression in gastric cells has not been addressed.

OCT-1 is a member of the POU homeodomain family of transcription factors [18] and is expressed ubiquitously. This protein recognizes the canonical octamer motif (ATGCAAAT) that regulates the transcription of various genes, such as the immunoglobulin genes in B cells [19], several interleukins

[20,21], and *Pit-1* [22]. It has been demonstrated that OCT-1 is implicated in the activation of the mouse *Cdx2* promoter in pancreatic and intestinal cell lines [23].

These observations prompted us to investigate whether OCT-1 could be inducing *CDX2* expression *de novo* in gastric mucosa, leading to the onset of IM. We addressed this issue by studying co-localization of CDX2 and OCT-1 proteins in gastric mucosa, intestinal metaplasia and gastric carcinomas. Furthermore, we evaluated OCT-1 binding by electrophoretic mobility shift assay and activation of the *CDX2* promoter by co-transfecting a *CDX2* promoter/reporter construct with an *OCT-1* expression vector into two gastric carcinoma cell lines, GP220 and MKN45.

Materials and methods

Tissue samples

For the study of OCT-1 expression in gastric mucosa and in intestinal metaplasia (IM), we used gastric biopsies from a cohort of asymptomatic or dyspeptic individuals from northern Portugal (Viana do Castelo), who volunteered to participate in the endoscopic examinations and biopsies [24]. Thirty-one biopsies had gastric mucosa with chronic gastritis without IM and 15 had IM foci in the setting of chronic gastritis. Biopsy samples were obtained according to the regulations of the local ethics committee and with informed consent of the patients. Forty-two gastric carcinomas were obtained from patients undergoing gastrectomy in Hospital S. João, Porto, Portugal. Carcinomas were histologically classified according to Laurén's classification [25]: 18 were intestinal, 15 were diffuse and 9 were atypical.

Immunohistochemistry

Paraffin-embedded samples were serially sectioned at 4 μm, mounted on gelatin-coated slides, dried overnight at 37 °C and dewaxed with xylene. OCT-1 staining was performed using an anti-OCT-1 polyclonal antibody (Labvision). Briefly, slides were boiled with an antigen retrieval solution (Vector) for 20 min, incubated with 3% hydrogen peroxide in methanol for 10 min, followed by incubation for 15 min with Ultra V block solution (Labvision), and finally incubated overnight at 4 °C with the primary antibody diluted 1 : 50 in 5% BSA (v/v). Subsequently, the UltraVision Detection System, anti-polyvalent HRP (Labvision) was used following the manufacturer's protocol. Slides were developed with DAB, counterstained with Mayer's haematoxylin, dehydrated and mounted with Entellan. Immunohistochemistry for CDX2 was previously performed in the same cases [10].

Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts from the gastric carcinoma cell lines GP202, GP220 and MKN45 were prepared as previously described [26] and kept at −80 °C until use. Protein content (2 μl of cell extracts) was measured using the bicinchoninic acid method (Pierce), as described in the manufacturer's instruction manual. The sequence of the probe (Proligo) used for the gel shift is as follows: 5′ TTTATCTTTTAAAATGCAAATTATGTTTCGAGGGG 3′. Radiolabelling of the probes was performed as previously described [14]. For supershift analysis, 1 μl of a monoclonal anti-OCT-1 antibody (Santa Cruz Biotechnology, USA) was added to the proteins and left for 30 min at room temperature before adding the radiolabelled probe. Electrophoresis and gel processing were as previously described [14].

Cell culture

Human gastric carcinoma cell lines GP220 (derived from a diffuse gastric carcinoma) and MKN45 (derived from a poorly differentiated gastric carcinoma) were cultured at 37 °C in a humidified 5% CO_2 incubator, maintained in RPMI 1640 medium (with Glutamax and 25 mM Hepes) supplemented with 10% fetal bovine serum and gentamycin (50 μg/ml). The colonic carcinoma cell line Caco2, used as a control, was cultured in DMEM under the conditions described above.

Transient transfection assays

Cell lines were seeded at 2.5×10^5/well in 24-well plates. Co-transfections were performed the next day by mixing 0.8 μg of the pGL3(−871/−1)Cdx2 promoter construct and 0.4 μg of the OCT-1 expression vector (a kind gift from Professor Winship Herr, Cold Spring Harbor Laboratory, NY) with Tfx-50 reagent (Promega) (Tfx-50 : DNA ratio of 4 : 1) in 200 μl serum-free medium. Cells were incubated with the transfection mixture for 1 h at 37 °C, followed by addition of 400 μl complete medium. Total cell extracts were prepared after 48 h incubation at 37 °C using 1× reporter lysis buffer (Promega), as described in the manufacturer's instruction manual. 20 μl cell extract were mixed with 100 μl luciferase assay reagent (Promega) to determine luciferase activity in a 1450 Microbeta luminescence counter (Wallac). β-galactosidase activity was measured using 50 μl cell extract.

Statistical analysis

The StatView 5.0 computer program was employed in the statistical analysis of data. Distributions were compared by χ^2 and Student's t-test and significance was assumed whenever p values were <0.05.

Results

Expression of OCT-1 in the gastric mucosa with and without IM and in gastric carcinomas

Expression of OCT-1 in gastric mucosa with chronic gastritis was observed in the nuclei of cells in the

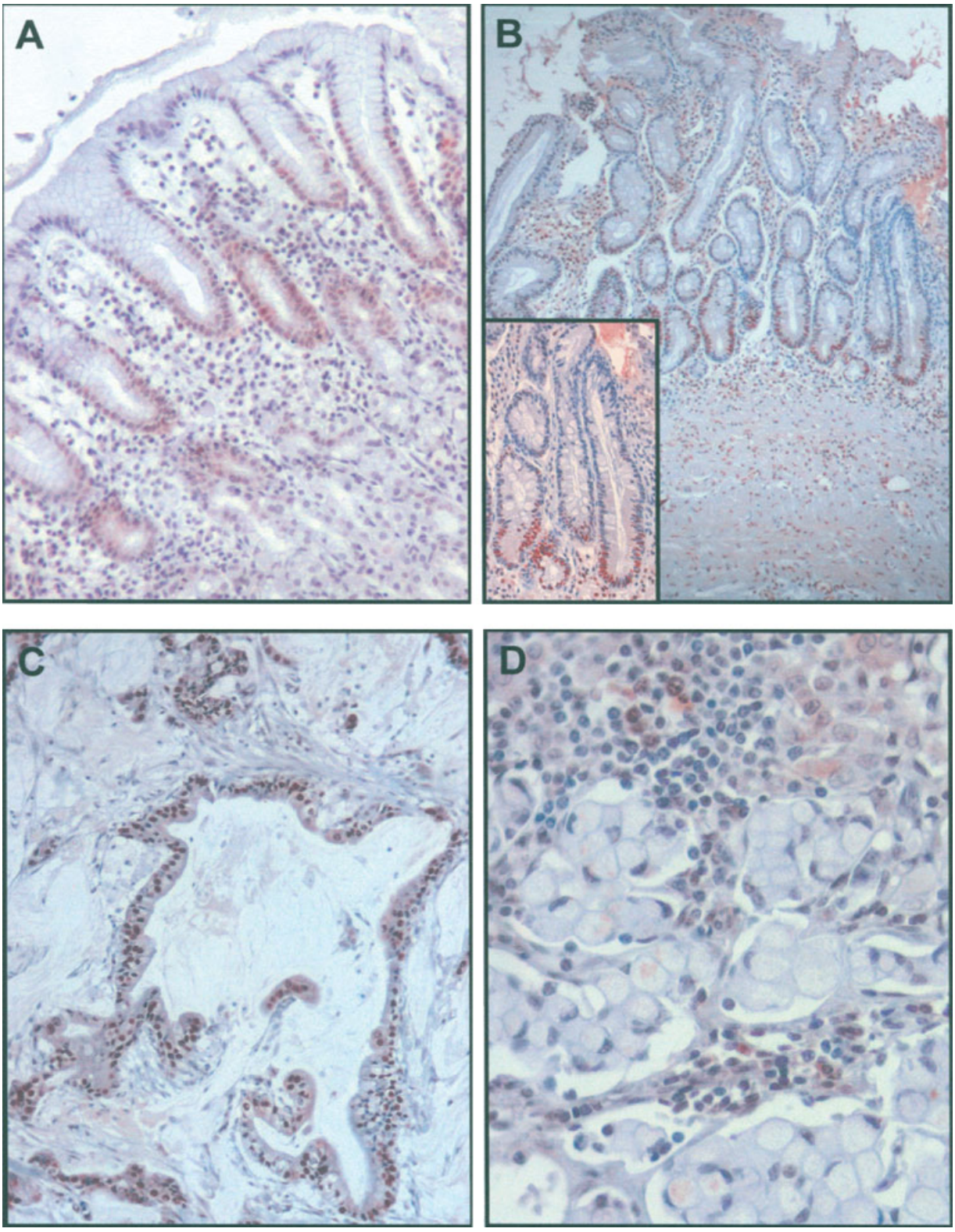

Figure 1. Gastric mucosa with and without intestinal metaplasia (IM) and gastric carcinomas immunostained for OCT-1. (A) OCT-1 is expressed in the neck region of the gastric mucosa without IM and is absent from the surface and the deep glands. (B) IM shows OCT-1 expression predominantly in the bottom of the crypts (insert in B). (C) Intestinal gastric carcinoma with OCT-1 expression and (D) diffuse gastric carcinoma without OCT-1 expression

proliferative region (Figure 1). OCT-1 was present in the gastric mucosa adjacent to IM in a high percentage of cases, 75% in the antrum, 71% in the incisura and 75% in the body region (Table 1), whereas in gastric mucosa without IM it was observed in a smaller percentage of cases, 22% in the antrum, 21% in the incisura and 38% in the body region (Table 1). IM lesions were positive for OCT-1 in 87% of the cases, in particular in the proliferative region, as observed in the normal gastric mucosa (Table 1, Figure 1). In all

Table 1. Expression of OCT-1 in 31 gastric mucosae with chronic gastritis, in 15 foci of IM and adjacent gastric mucosae, obtained from the antrum, incisura and body regions of the stomach

	Mucosa without IM						Mucosa with IM	
	Antrum		Incisura		Body			
	+ (%)	− (%)	+ (%)	− (%)	+ (%)	− (%)	+ (%)	− (%)
Cases with IM	3 (75)	1 (25)	5 (71)	2 (29)	3 (75)	1 (25)	13 (87)	2 (13)
Cases without IM	2 (22)	7 (78)	3 (21)	11 (79)	3 (38)	5 (63)	—	—

Table 2. Expression of OCT-1 in a series of gastric carcinomas, according to Laurén's classification and to the expression of CDX2

	OCT-1		
Parameters	**Negative *n* (%)**	**Positive *n* (%)**	***p* Value**
Laurén's classification ($n = 42$)			
Intestinal ($n = 18$)	3 (17)	15 (83)	
Diffuse ($n = 15$)	8 (53)	7 (47)	
Atypical ($n = 9$)	0 (0)	9 (100)	0.008
CDX2 expression ($n = 42$)			
Positive ($n = 23$)	6 (26)	17 (74)	
Negative ($n = 19$)	5 (26)	14 (74)	n.s.

cases some inflammatory cells were also positive for OCT-1.

We also analysed OCT-1 expression in 42 gastric carcinomas. Overall, 74% of the gastric carcinomas were positive for OCT-1. We could not identify an association between OCT-1 expression and CDX2 expression in our series of carcinomas (Table 2). However, we observed a strong association between OCT-1 expression and the histological type of gastric carcinoma (Table 2). The majority of the gastric carcinomas of the intestinal type were positive for OCT-1 (83%), whereas 53% of the diffuse gastric carcinomas were negative (Table 2, Figure 1).

EMSA

We performed EMSA to evaluate whether OCT-1 binds to the OCT-1 consensus sequence present in the *CDX2* promoter (Figure 2A). Incubation of the *OCT-1* probe located at −416/−409 with nuclear proteins from GP220, GP202 and MKN45 gastric carcinoma cell lines led to the formation of three complexes (Figure 2B), which were shown to be specific, since pre-incubation with 50× molar excess of the cold probe resulted in the absence of retarded bands (Figure 2B). Binding of OCT-1 was confirmed by a total supershift of complex 1 upon addition of anti-OCT-1 antibody in the reaction mixture (Figure 2B).

Role of the transcription factor OCT-1 on CDX2 promoter activity in gastric and colonic carcinoma cell lines

We co-transfected MKN45, GP220 and Caco2 cell lines with the *CDX2* promoter construct −871/−1 and a vector encoding OCT-1 (pCG−OCT-1). We could not observe transactivation of the *CDX2* promoter by the OCT-1 transcription factor in the gastric cell lines (Figure 2C). The Caco2 colonic cell line showed higher OCT-1 transactivation when compared to gastric cell lines (Figure 2C).

Discussion

We observed that OCT-1 is expressed in non-metaplastic gastric mucosa in the proliferative region, and that its expression is increased in the mucosa adjacent to IM foci. We further identified OCT-1 expression in 87% of IM foci, mostly in the proliferative region, where it co-localized with CDX2. These observations support our hypothesis that OCT-1 might trigger aberrant expression of *CDX2* in gastric cells, thus leading to an intestinal transdifferentiation process. Our initial assumption was based on several previous studies: OCT-1 transactivates the mouse *Cdx2* promoter in the intestinal cell line Caco-2 and in the pancreatic cell line InR1-G9 [23]. Furthermore, OCT-1 protein expression is increased in response to genotoxic stress in multiple human cell lines [27]. Therefore, we hypothesized that OCT-1 might be up-regulated in an inflammatory setting such as chronic atrophic gastritis, where IM develops. The increased OCT-1 expression in IM and in gastric mucosa in the vicinity of IM is thus in agreement with our hypothesis. However, the presence of OCT-1 in the proliferative region of non-metaplastic mucosa with chronic gastritis suggests that it is not, at least by itself, sufficient to trigger *CDX2* expression.

In gastric carcinoma cases we did not observe a significant association between OCT-1 and CDX2 expression: OCT-1 is observed in 76% of the carcinomas, whereas CDX2 is observed in 55% [10]. This suggests that CDX2 expression might be lost during progression of gastric carcinomas from IM. IM is considered a risk factor for gastric carcinoma, based on the results of various epidemiological studies [3] and it has recently been shown that transgenic mice that express CDX2 ectopically in the stomach, in addition to developing IM [16,17], later develop gastric polyps with features of invasive carcinomas [28]. The significant association of OCT-1 with intestinal-type gastric carcinomas as well as with IM suggests that, directly or via CDX2, OCT-1 is playing a role in gastric carcinogenesis that

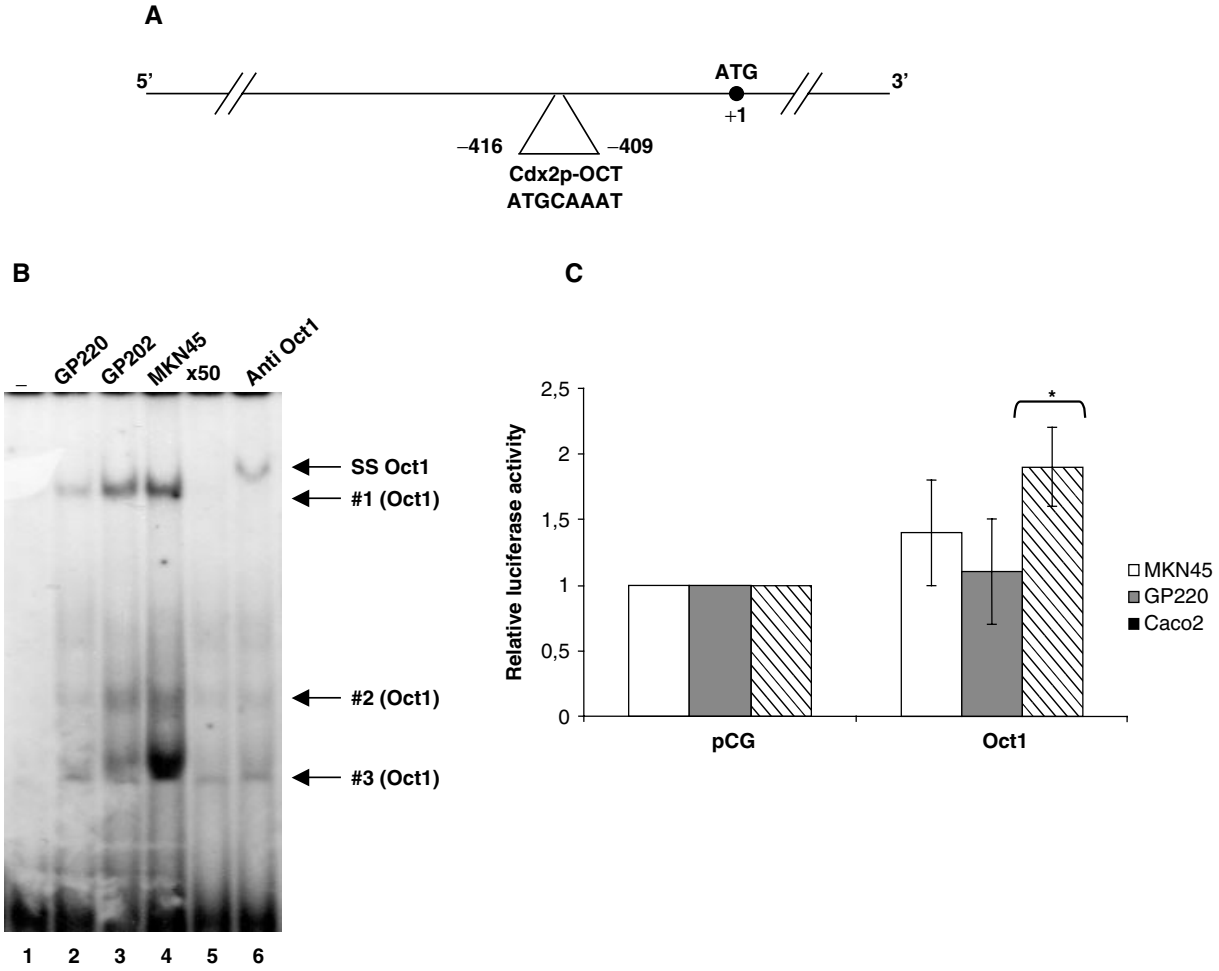

Figure 2. Study of the involvement of OCT-1 in the regulation of *CDX2* promoter activity. (A) Schematic representation of the OCT-1 putative binding site at −416/−409 in the *CDX2* promoter region. (B) EMSA performed with nuclear extracts from GP220, GP202 and MKN45 gastric carcinoma cell lines and an OCT-1 radiolabelled probe. Lane 1 is the probe alone; lanes 2–4 are the nuclear extracts incubated with the radiolabelled probe; lane 5 is the competition assay performed with 50× excess of cold probe; and lane 6 is a supershift assay performed with an antibody against OCT-1. DNA–protein complexes and the supershifted complex (SS Oct1) are indicated by arrows. (C) Regulation of *CDX2* promoter activity in the gastric carcinoma cell lines MKN45 and GP220 and in the intestinal cell line Caco2 by OCT- 1. *CDX2* promoter construct −871/−1 was co-transfected with pCG-OCT-1 expression vector and with the empty vector pCG for control. The values obtained in cells transfected with the empty vector are referred to as 1. The results are means ± SD and represent two separate experiments done in triplicate. $^{*}p < 0.02$

leads to intestinal-type gastric carcinomas. Loss of CDX2 expression in gastric carcinomas that arise from IM is in agreement with the fact that CDX2 expression decreases in human colorectal cancers in relation to tumour grade and is lost in minimally differentiated colon carcinomas [29,30].

In an attempt to clarify the capacity of OCT-1 to transactivate *CDX2* in gastric cells, we performed luciferase and EMSA studies that showed that, despite having the capacity to bind to the *CDX2* promoter, OCT-1 does not efficiently transactivate *CDX2* in our model. This observation is in contrast to what was previously described in colonic and pancreatic cell lines [23] and the same trend was observed in the present study in Caco2. The results we obtained in gastric cell lines may stem from cell-type specificity in the regulation of *CDX2* by OCT-1 due to the participation of co-factors. In fact, and lending support to this interpretation, OCT-1 is known to interact with co-factors to activate different proteins, such as with STAT5 in the activation of cyclin D1 [31] and with Bob1 to stimulate transcription of immunoglobulin genes [32].

In conclusion, we observed significantly increased OCT-1 expression in IM and in intestinal gastric carcinomas and identified the capacity of OCT-1 to bind to the *CDX2* promoter, although we could not demonstrate a direct effect of OCT-1 in the transactivation of CDX2.

Acknowledgements

This work was supported by Fundação para a Ciência e Tecnologia, Programa Operacional Ciência, Tecnologia e Inovação do Quadro Comunitário de Apoio III (Project POCTI/CBO/39075/2001) and the European funding FEDER, by Fundação Calouste Gulbenkian (Project FC-54918) and by Fundação Luso-Americana para o Desenvolvimento (Project 173/202).

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